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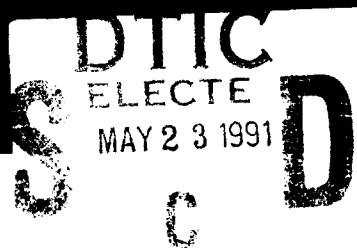
A CTL epitope on the circumsporozoite protein of *P. yoelii*

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Humans are infected with malaria by the bite of anophelene mosquitos carrying plasmodia sporozoites. These sporozoites pass quickly from the blood into hepatocytes, where they develop into mature liver-stage parasites over several days. The clinical stage of the illness begins only when the liver-stage parasites rupture into the bloodstream and erythrocytes are invaded. The pre-erythrocytic stages of malaria are inviting targets for vaccine development, because an effective immune response to these early stages would prevent symptomatic infections.

Introduction

A vaccine model exists for protection against the pre-erythrocytic stages of malaria. Humans and rodents can be protected against sporozoite infection by immunization with radiation-attenuated sporozoites (1, 2). There is evidence from different animal models that both antibodies and T cells may be involved in this immune response (3, 4). However, when BALB c mice are immunized with *Plasmodium yoelii* sporozoites, it appears that antibody alone plays a minor protective role, while CD8⁺ T cells have critically important anti-sporozoite functions (4). The parasite antigens recognized by these protective CD8⁺ T cells have not been identified. The circumsporozoite (CS) protein is as yet the only well characterized parasite antigen from the pre-erythrocytic stages of *P. yoelii* (Fig. 1); it is a 368-aminoacid protein whose sequence has been defined at the DNA level (5). We wished to know if the *P. yoelii* CS protein was a target of immune CD8⁺ T cells. We now report the discovery and characterization of an epitope for cytotoxic CD8⁺ T lymphocytes (CTL) on this parasite protein.

Methods and results

Screening for CTL activity with synthetic peptides. The 26 peptides spanning the *P. yoelii* CS protein were divided into 5 sets, 1–6, 7–11, 12–16, 17–21,

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and 22–26. Spleen cells from sporozoite-immunized BALB c mice were stimulated with one of the 5 peptide sets and RCAS for 7 days. Each individual peptide within a set was at 5 micromolar concentration. Cultured cells stimulated by each peptide set were harvested and used in chromium-release assays against target cells labelled with a single peptide from that pool.

Effector : target ratios were 40:1 and the background lysis of P815 cells without peptide was determined for each set of cultures. Peptide 19 (281–300), and to a lesser extent peptide 18 (271–290), stood out as the only peptides labelling targets for lysis (Fig. 2).

Stimulation with individual peptides. To follow up the results of screening, spleen cells from immune animals were cultured with individual peptides. When peptide 18 alone was used to stimulate cultures and to label targets no significant lysis was detected (data not shown). However, culture with peptide 19 caused the effector cells to lyse peptide-labelled target cells. Further experiments showed that CTL activity was not produced unless both peptide 19 and RCAS were included in the medium. EL4 (H-2ᵇ) targets cultured with peptide 19 were not lysed by the BALB/c (H-2ᵈ) effector cells. Treatment with anti-CD8 monoclonal antibody (MAb) and complement completely removed lytic activity from cell cultures. Normal BALB/c spleen cells would not lyse targets after *in vitro* stimulation. In addition, BALB/c mice immunized with another malaria parasite, *P. berghei*, would not lyse targets after stimulation with *P. yoelii* peptide 19 (Fig. 3).

Fine mapping of the CTL epitope. A nested set of peptides was made by deleting single aminoacids from amino- and carboxy-ends of the peptide-19 sequence.

Fig. 1. **A diagram showing the structure of the** *P. yoelii* **CS protein** (5). Solid blocks show constant regions RI and RII. Shaded blocks show the areas of the 3 repeat motifs. The locations of the 26 synthetic peptides used for screening are marked. Each peptide with the exception of the last was 20 aminoacids long and overlapped its neighbours by 10 aminoacids.
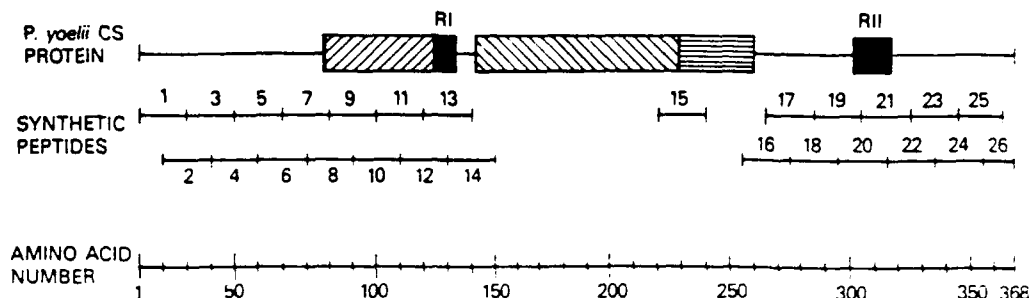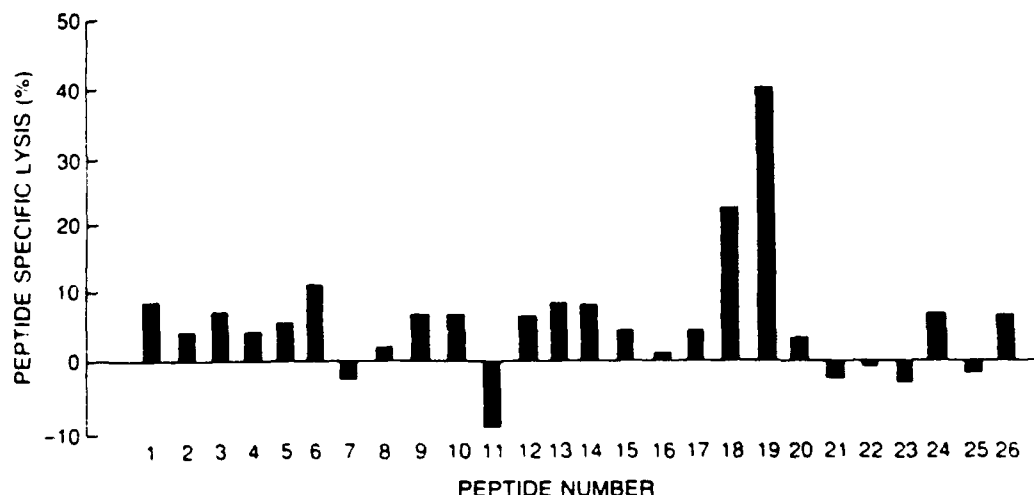

Fig. 2. **Immune spleen cells were cultured with synthetic peptides (see text), and cultured cells were used as effector cells against P815 targets labelled with individual peptides.** Effector: target ratios were 40 1

Sporozoite-immunized BALB c spleen cells were cultured with peptide 19 and then tested against P815 targets incubated with each of the nested peptides at several concentrations (Fig. 4). Shortening by a single amino-terminal residue at position 281 reduced labelling, and shortening by two aminoacids abolished labelling activity. Lengthening peptide 19 by one aminoacid at position 280 actually decreased labelling activity. At the carboxy-terminal, truncated peptides had full activity until the isoleucine at position 296 was removed. Complete loss of labelling occurred with deletion of the valine at position 293. Thus a 12-aminoacid core is essential for target labelling, but optimal labelling requires a 16-amino-
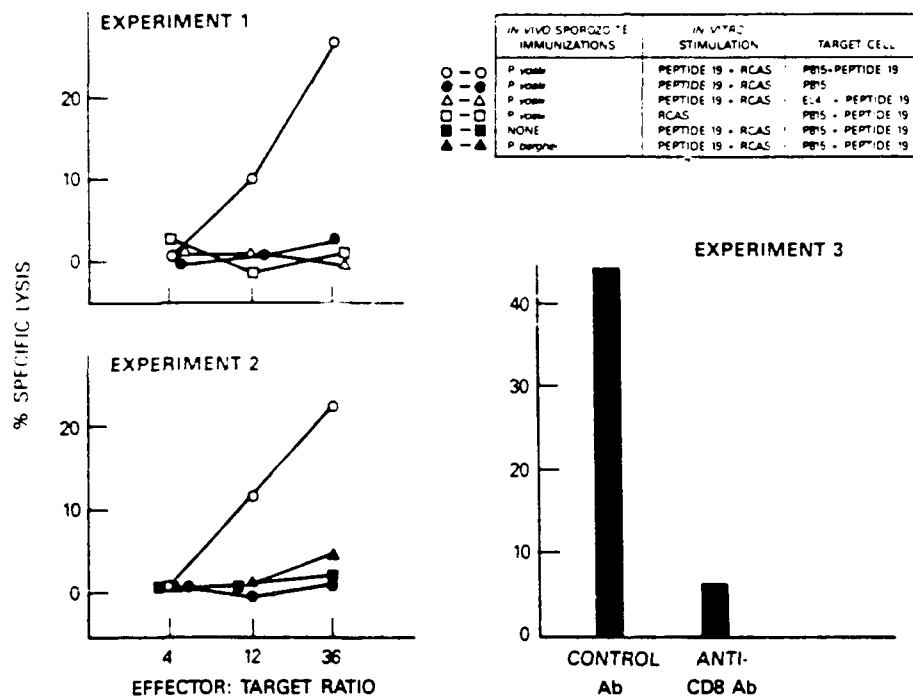
acid peptide containing one additional amino-terminal and three additional carboxy-terminal residues (Fig. 5).

Discussion

Using synthetic peptides based on DNA sequences, we have discovered an epitope recognized by CTL on the *P. yoelii* CS protein. We have mapped a 12-aminoacid minimal core required for labelling targets for lysis, and four adjacent aminoacids which boost labelling to maximal levels. In our initial screening experiments, a 20-aminoacid peptide (281–300) containing this epitope was the most effective of all

Fig. 3. *Experiment 1*: Immune spleen cells were cultured with RCAS alone, or with RCAS and peptide 19. Targets in the Cr-51 release assay were P815 (H-2^d) cells alone, P815 cells with peptide 19. or EL-4 (H-2^b) cells with peptide 19.
Experiment 2: Mice were immunized with either 2 doses of *P. yoelii* sporozoites or *P. berghei* sporozoites. Spleen cells from each group as well as from unimmunized mice were cultured *in vitro* with peptide 19 and RCAS. Target cells in the Cr-51 release assay were P815 cells alone, and P815 cells with peptide 19.
Experiment 3: Immune spleen cells were cultured with peptide 19 and RCAS. Cells were incubated with targets at a 30:1 effector:target ratio in the presence of anti-CD8 MAb or control Ig at a concentration of 0.1 mg/ml.

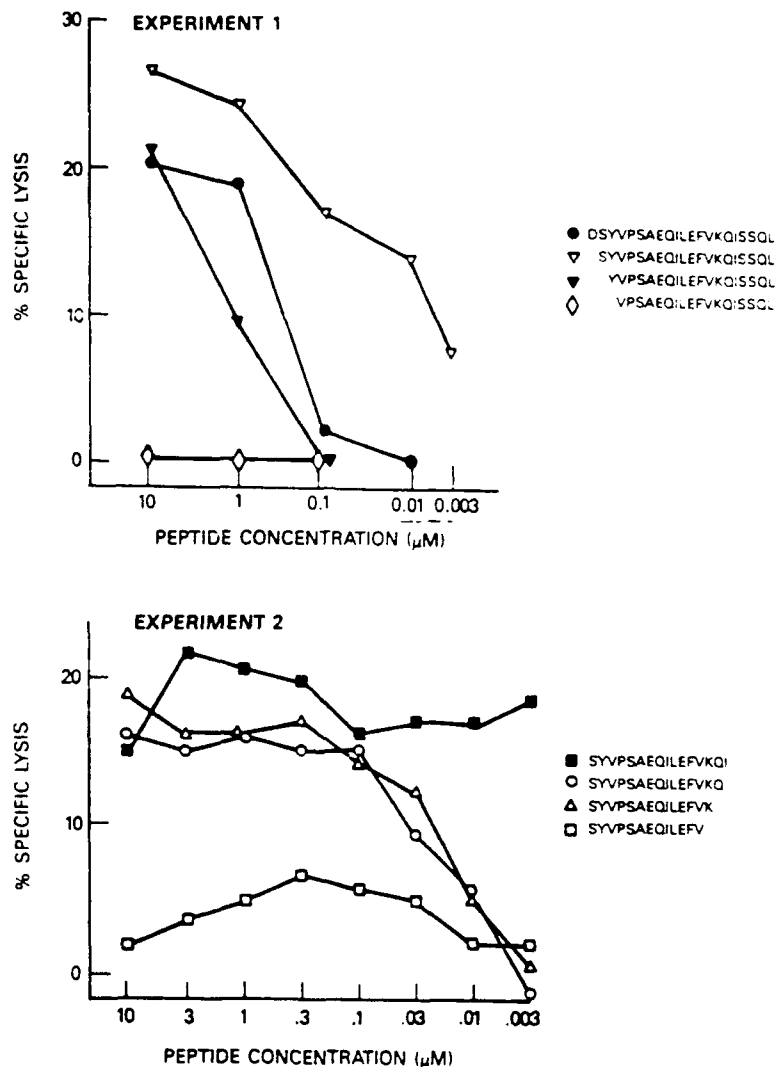| IN VIVO SPOROZOITE IMMUNIZATIONS	IN VITRO STIMULATION	TARGET CELL
O — O P. yoelii	PEPTIDE 19 + RCAS	P815+PEPTIDE 19
● — ● P. yoelii	PEPTIDE 19 + RCAS	P815
△ — △ P. yoelii	PEPTIDE 19 + RCAS	EL4 + PEPTIDE 19
□ — □ P. yoelii	RCAS	P815 + PEPTIDE 19
■ — ■ NONE	PEPTIDE 19 + RCAS	P815 + PEPTIDE 19
▲ — ▲ P. berghei	PEPTIDE 19 + RCAS	P815 + PEPTIDE 19

overlapping peptides at labelling targets for lysis. Subsequent experiments showed that this peptide was able to stimulate immune cells to become CTL *in vitro*, and to label targets for lysis by these cells. The neighbouring peptide (271-290), containing 10 of the 12 core aminoacids of this epitope, had some reduced ability to label targets but was unable to stimulate immune cells to have cytotoxic activity. Therefore we do not believe that this activity in peptide (271-290) represents a separate epitope in BALB/c mice.

Synthetic peptides provided an easy means of defining this T epitope. Our attempts using other methods such as vaccinia virus recombinants or transfected cells have been hampered by technical difficulties. There are two complementary strategies for using synthetic peptides to find T-cell epitopes. The first is to synthesize peptides chosen by predictive algorithms (9, 10). Our peptide 19 was predicted by the AMPHI program to fold as an amphipathic alpha-helix, and was a T-epitope candidate on that basis. The second strategy is to empirically produce peptides which are screened for reactivity with T cells. Typically, it is not practical to synthesize all possible peptides, and overlapping peptides are used. This works well if T-cell epitopes are as short as 8 or 9 aminoacids but such screening is difficult for longer T-cell epitopes. Our series of overlapping peptides, 20 aminoacids long overlapping by 10, reproduces all possible sequences of 11 aminoacids or shorter. However, 10% of all possible 12 aminoacid sequences are missed, and this percentage increases with sequence length such that only 50% of all 16 aminoacid sequences are contained in our peptide set. Our core CTL epitope has 12 aminoacids, and 16 aminoacids are necessary for optimal activity. We were fortunate to have found our epitope using these peptides.

Fig. 4. *Experiment 1.* Immune spleen cells were cultured with peptide 19 (281–300) and RCAS and used at an effector : target ratio of 30:1. P815 cell targets were incubated with Cr-51 overnight, and 1 of 4 nested amino-terminal peptides was added at several concentrations during the chromium release assay.
Experiment 2. Immune spleen cells were cultured with peptide 19 (281–300) and used at a 30:1 effector : target ratio. P815 cell targets were incubated with Cr-51 overnight, and 1 of 4 peptides sequentially shortened from the carboxy-terminal was added at several concentrations during the chromium release assay.

EXPERIMENT 1

● DSYVPSAEQILEFVKQISSQL
▽ SYVPSAEQILEFVKQISSQL
▼ YVPSAEQILEFVKQISSQL
◊ VPSAEQILEFVKQISSQL

EXPERIMENT 2

■ SYVPSAEQILEFVKQI
○ SYVPSAEQILEFVKQ
△ SYVPSAEQILEFVK
□ SYVPSAEQILEFV

The CTL we have demonstrated were generated by immunization with the whole sporozoite. and CTL activity was not inducible in spleen cells from normal BALB/c mice or from mice immunized with *P. berghei.* a closely related rodent malaria parasite. Interestingly, the region of the *P. berghei* CS protein (*11*) homologous to our 281–296 *P. yoelii* epitope has different aminoacids at two of the 12 core positions

Fig. 5. **The aminoacid sequence 281–296 from the *P. yoelii* CS protein.** The 16 aminoacids are required for maximum target labelling, while the 12 aminoacid core sequence is shown by the box. Below is the homologous region from the *P. berghei* CS protein. The two differences in the core region are highlighted by asterisks.

```
        281                              296
P.yoelii CS  S │Y V P S A E Q I L E F V K│ Q I

P.berghei CS  S Y I P S A E K I L E F V K Q I
                  •       •
        183                              199
```

(Fig. 5). These may be altering the binding to MHC I molecules or the T-cell receptor. Carbone et al. *(12)* have described induction of CTL in normal spleen cells using synthetic peptides *in vitro*. However, they cultured cells at extremely high cell density, and we see no evidence of *in vitro* induction of CTL in cells from control animals using our lower density cultures.

Kumar et al. *(13)* have previously defined a CTL epitope recognized by mouse cells on the CS protein of the human parasite *P. falciparum*. However, mice cannot be infected with this parasite and the relationship of this CTL epitope to protective immunity has not been tested. With the discovery of a CTL epitope in a rodent malaria parasite, we should be able to understand the role of these CTL in the protective immune response.

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